

OTHER VIRUS DISEASES

Virus Content and Growth Patterns of Callus Cultured *in vitro* from Healthy and Virus-Infected Citrus Species

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ABSTRACT. Stem segments from healthy and virus-infected Pineapple sweet orange, Mexican lime and Etrog citron were cultured on callus-inducing medium. Explants infected with citrus infectious variegation virus (CIVV), psorosis, vein enation and cachexia produced significantly less primary callus than healthy controls, whereas citrus tristeza virus (CTV) did not affect callus production. The growth of secondary callus during subculturing was similar in healthy and infected tissues. Indexing of callus indicated that the number of calli infected with CTV and vein enation decreased drastically with subculturing, whereas the number of calli infected with CIVV and psorosis did not decrease after five subcultures.

Index words. callus indexing, tristeza, infectious variegation, psorosis, vein enation, cachexia-xyloporosis.

The potential of using plant tissue cultures for the study of plant viruses was recognized by White in 1934 (27). During the 1940's and early 1950's, several attempts were made to use callus cultures from tomato and tobacco tissues infected with tobacco mosaic virus (TMV) (2, 12, 20). However, estimates of virus content by serology or by a local lesion assay, left the general perception that tissue cultures had low virus titers (2, 12, 21, 22) which limited their usefulness for virus research.

During the late 1950's and 1960's, with the availability of carefully defined nutrient media (5, 14) and a better understanding of the role of plant growth regulators on tissue culture media (24), the establishment of long-term cultures from a number of virus-plant host combinations was repeatedly attempted (6, 7, 9, 15, 16, 18). However, in spite of numerous efforts, most review articles reflected some disappointment in the results achieved (8, 28, 29).

Recently, callus and cell suspension cultures have been useful for viroid studies (13, 30), including the citrus exocortis viroid (10, 11, 26). Vir-

oid-containing cultures were easy to manipulate and maintained stable viroid titers after periodical subculturing, for extended periods of time. In view of these results, we questioned again the usefulness of tissue cultures for virus research.

This report describes the results of a survey study done to investigate the growth patterns and virus content of callus cultures established from a number of healthy and virus-infected citrus hosts. The diseases studied were tristeza, infectious variegation, psorosis, vein enation and cachexia (xyloporosis). The hosts utilized were Pineapple sweet orange, Mexican lime, and Etrog citron Arizona 861-S-1.

DISEASE ISOLATES AND HOSTS

Citrus Tristeza Virus (CTV).

Two isolates of CTV, coded as T-300 and T-308, were utilized. Isolate T-300 induces mild symptoms on Mexican lime and it was obtained by exposing Mexican lime seedlings to natural infection in the field. This isolate is representative of the common type of CTV found in Spain. Isolate T-308 induces severe symptoms on Mexican

lime including stunting, vein clearing, leaf yellowing, stem pitting and sometimes vein corking, but it does not induce the seedling yellows reaction. This isolate was obtained from a calamondin tree of unknown origin. Both isolates were known to be free from other virus and viruslike diseases. For tissue culture studies, inoculated Pineapple sweet orange and Mexican lime seedlings were used.

Citrus Infectious Variegation Virus (CIVV). A single isolate (IV-400), originally from Corsica, was utilized. It produces characteristic leaf distortion, wrinkling, flecking and chlorotic spots on inoculated sweet orange and citron plants. This isolate tested negative for tristeza, cachexia and exocortis. Inoculated Pineapple sweet orange seedlings and Arizona 861-S-1 citrons budded on sour orange were used for tissue culture studies.

Psorosis. Two isolates of psorosis, coded as P-121 and P-123, were utilized. Both isolates were obtained from a single Oroval clementine source which produced a shock reaction on inoculated Pineapple sweet orange seedlings and tested negative for tristeza and vein enation and positive for exocortis and cachexia. Two clementine plants obtained by shoot-tip grafting *in vitro* produced two distinct reactions when inoculated on Pineapple sweet orange seedlings. The source coded P-121 caused a shock reaction, vein flecking, oak-leaf patterns and crinkling characteristics of the original isolate. In contrast, P-123 only induced flecking and oak-leaf patterns. Both P-121 and P-123 isolates are believed free of exocortis and cachexia. The isolate P-121 protected inoculated plants against challenge inoculation with psorosis lesion bark inoculum, whereas isolate P-123 did not. Inoculated Pineapple sweet orange seedlings were used for the establishment of tissue cultures.

Vein Enation. A single isolate (VE-204), which induces characteristic enations on veins was utilized. This isolate was originally found on

Parent Washington navel which tested negative for tristeza, psorosis, cachexia and exocortis. Inoculated Pineapple sweet orange seedlings were used as source of tissue.

Cachexia. A single isolate (X-704), which originally came from California (CA-902) and induces a severe reaction on Parson's Special mandarin grafted on rough lemon rootstock, was utilized. This isolate tested negative for tristeza, psorosis and exocortis. Inoculated Pineapple sweet orange seedlings and Arizona 861-S-1 citrons grafted on rough lemon were used for tissue culture studies.

INDUCTION OF CALLUS

Stem segments from healthy and infected plants were utilized as tissue explants for initiation of *in vitro* cultures. In all instances, the presence of pathogens in source plants was confirmed by indexing or direct observation of characteristic symptoms of the disease. Stem pieces (20 cm long) were stripped of leaves and thorns, disinfected by immersion for 20 min in a 2% sodium hypochlorite solution containing 0.1% Tween 20 wetting agent and rinsed three times with sterile water. Explants were prepared by excising 1-cm-long internode segments which were bisected longitudinally (fig. 1A). They were cultured by placing the longitudinal cut surface in contact with the culture medium (fig. 1B) which contained the inorganic salts of Murashige and Skoog (14), 100 mg/l *i*-inositol, 0.2 mg/l thiamine hydrochloride, 1 mg/l pyridoxine hydrochloride, 1 mg/l nicotinic acid, 0.25 mg/l 6-benzylamino purine, 10 mg/l naphthalene acetic acid, 30 g/l sucrose and 10 g/l agar. The pH was set at 5.7 ± 0.1 and the media autoclaved at 121 C for 15 min. The cultures were kept in darkness at 25-27 C.

Proliferation of primary callus (fig. 1C) from the cut surface was first observed 2 weeks after the cultures had been initiated. The growth of pri-

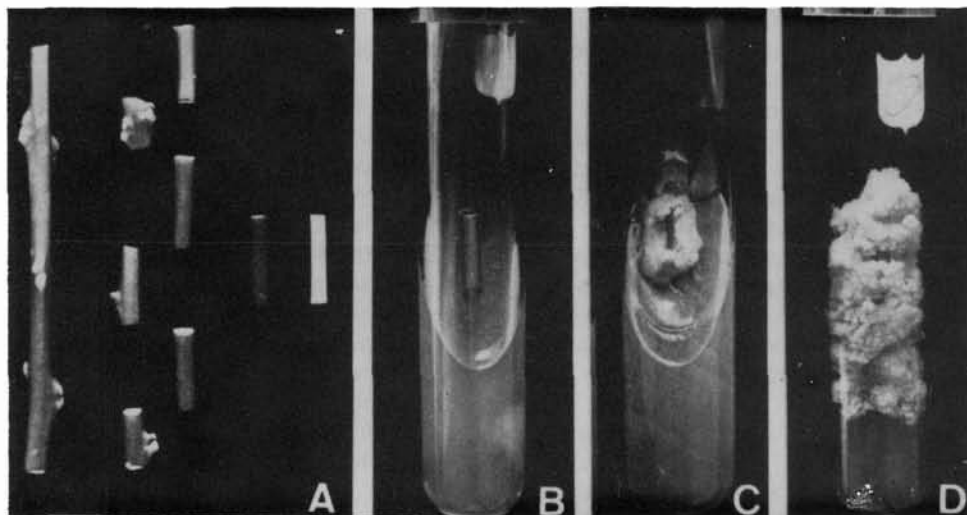


Fig. 1. Induction and culture of callus. A) preparation of tissue explants from stem pieces B) freshly made culture of a stem segment C) primary callus after 3 weeks in culture D) callus maintained by periodical subculture, 8 weeks after the last subculture.

mary callus continued during the following 3 weeks until the original explant was virtually covered by callus.

Minor differences in color and texture were observed among the calli from healthy controls of the three species studied. However, the primary callus from infected tissues had the same general aspect as the controls obtained from healthy tissues of the same citrus species. Callus production was not affected by CTV infection of the initial explant, whereas tissues infected with CIVV or vein enation produced significantly less primary callus than their healthy controls (table 1). Although the production of primary callus from psorosis-infected tissues did not differ from the healthy control, significant differences were obtained between the two isolates used (table 1). The effect of cachexia infection was species dependent. Cachexia-infected Pineapple sweet orange produced significantly less primary callus than the control, whereas cachexia-infected Arizona 861-S-1 citron produced significantly more than its control (table 1).

GROWTH PATTERNS OF CALLUS CULTURES

After 5 weeks in culture the callus produced by the initial explant was excised and transferred to fresh medium. The medium for callus subculture had the same composition as the medium utilized to culture the initial explant, supplemented with 10% (v:v) orange juice. After 4 weeks in culture under the same environmental conditions described for initiation of primary callus, a significant growth of secondary callus was observed.

Secondary callus originated from healthy and infected explants were maintained for several months by subculturing part of the newly produced callus (approx. 50 mg) every 4 weeks. The cultures, derived from infected explants and maintained by periodical subcultures, had the same color, texture and gross morphology as the healthy controls of the same species.

At the end of each 4-week culture period, and prior to subculture, the calli were weighed to evaluate callus growth on long-term cultures. When the mean weights of the different cal-

TABLE 1
INDUCTION OF PRIMARY CALLUS ON HEALTHY AND INFECTED CITRUS EXPLANTS
CULTURED *IN VITRO*

Source of tissue		Isolate no.	Production of ^z primary callus (g)
Disease	Host		
Tristeza (CTV)	Pineapple sweet orange	Control	0.78 a
		T-300	0.83 a
		T-308	0.83 a
	Mexican lime	Control	0.86 a
		T-300	0.74 a
		T-308	0.84 a
Infectious Variegation (CIVV)	Pineapple sweet orange	Control	0.51 a
		IV-400	0.33 b
	Arizona 861-S-1 citron	Control	0.42 a
		IV-400	0.30 b
Psorosis	Pineapple sweet orange	Control	1.07 ab
		P-121	1.28 a
		P-123	1.00 b
Vein Enation	Pineapple sweet orange	Control	1.56 a
		VE-207	1.18 b
Cachexia	Pineapple sweet orange	Control	0.54 a
		X-704	0.38 b
	Arizona 861-S-1 citron	Control	1.44 a
		X-704	1.66 b

^zThe production of primary callus was evaluated by weighing the cultures 5 weeks after initiation. Values represent the mean weight of at least 20 cultures. Means followed by the same letter are not significantly different (0.05 probability level). Statistical analysis was independent for each host/disease combination.

lus lines were plotted on a graph, characteristic growth patterns were obtained (figs. 2 to 6). The callus lines derived from infected tissues presented the same general growth patterns as the healthy controls. The statistical analysis (0.05 probability level) of the weights of the cultures at the end of each subculture period showed only minor differences between the growth of callus lines from healthy and infected tissues.

The production of callus from CTV-infected tissues did not differ from the healthy controls in the four subcultures studied (fig. 2). Conversely, the low production of primary callus from CIVV-infected tissues (table 1) was partially retained during the following subcultures. Pineapple sweet orange callus de-

rived from CIVV-infected plants grew less than the healthy controls during the first and second subcultures (fig. 3), whereas no differences were found over seven subcultures between healthy and infected callus lines of Arizona 861-S-1 citron.

Although tissues infected with vein enation and cachexia differed from healthy controls in the production of primary callus, no significant differences in growth were found (figs. 4 and 5) during subculturing. The psorosis-infected callus lines followed a different pattern; they did not differ from the healthy controls during first, second and third subcultures, but the P-123 infected lines grew significantly less than the healthy controls on the fourth and fifth subcultures (fig. 6).

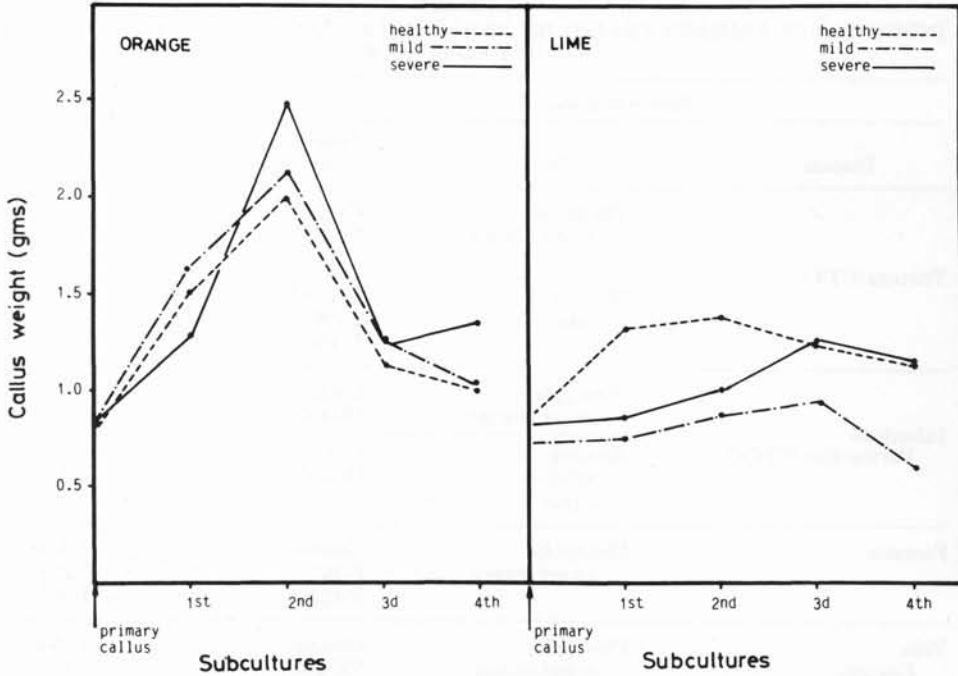


Fig. 2. Growth patterns of callus lines obtained from stem segments of healthy and tristeza (mild, T-300 and severe, T-308 strains) infected Pineapple sweet orange and Mexican lime plants. The plotted data represent the mean weight of at least 15 cultures at the end of each subculture period.

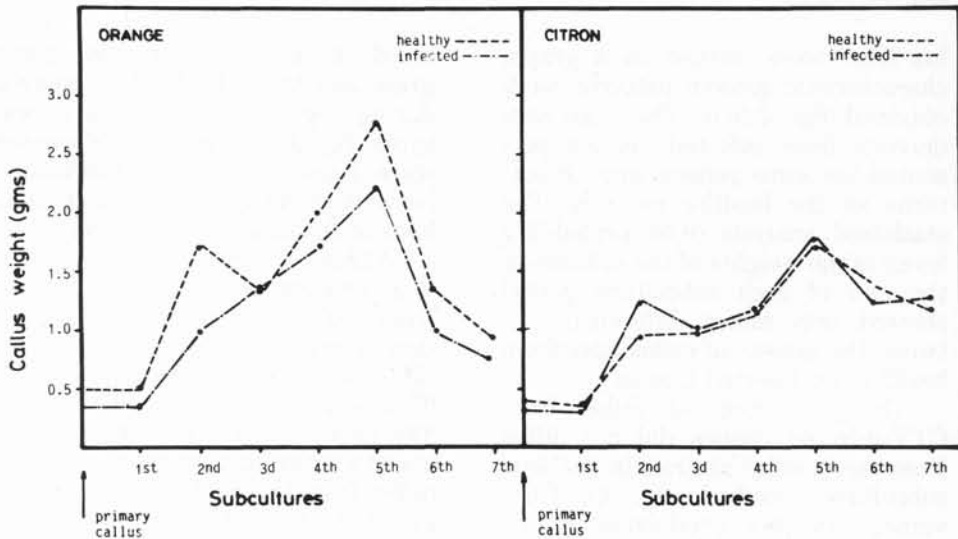


Fig. 3. Growth patterns of callus lines obtained from stem segments of healthy and infectious variegation (IV-400) infected Pineapple sweet orange and Arizona citron 861-S-1 plants. The plotted data represent the mean weight of at least 15 cultures at the end of each subculture period.

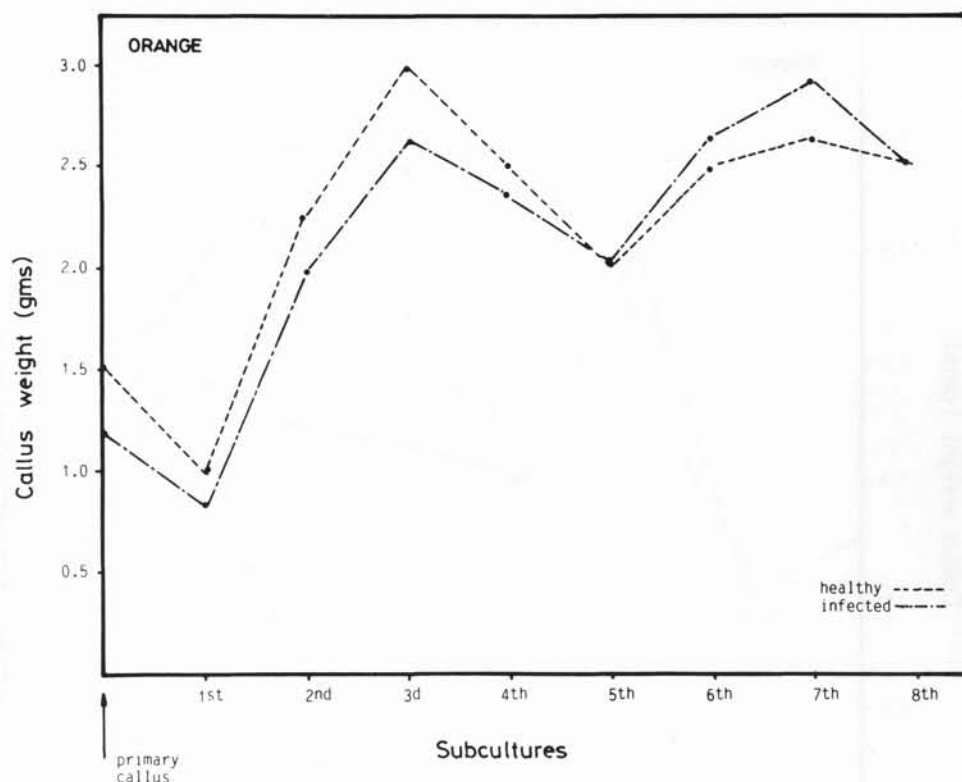


Fig. 4. Growth patterns of callus lines obtained from stem segments of healthy and vein enation (VE-207) infected Pineapple sweet orange plants. The plotted data represent the mean weight of at least 15 cultures at the end of each subculture period.

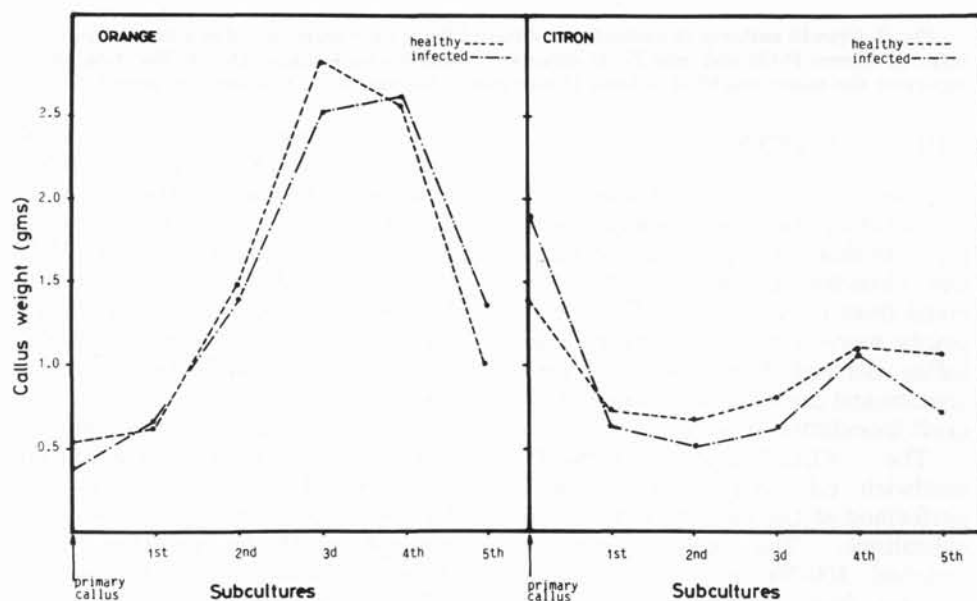


Fig. 5. Growth pattern of callus lines obtained from stem segments of healthy and cachexia (X-704) infected Pineapple sweet orange and Arizona citron 861-S-1 plants. The plotted data represent the mean weight of at least 15 cultures at the end of each treatment.

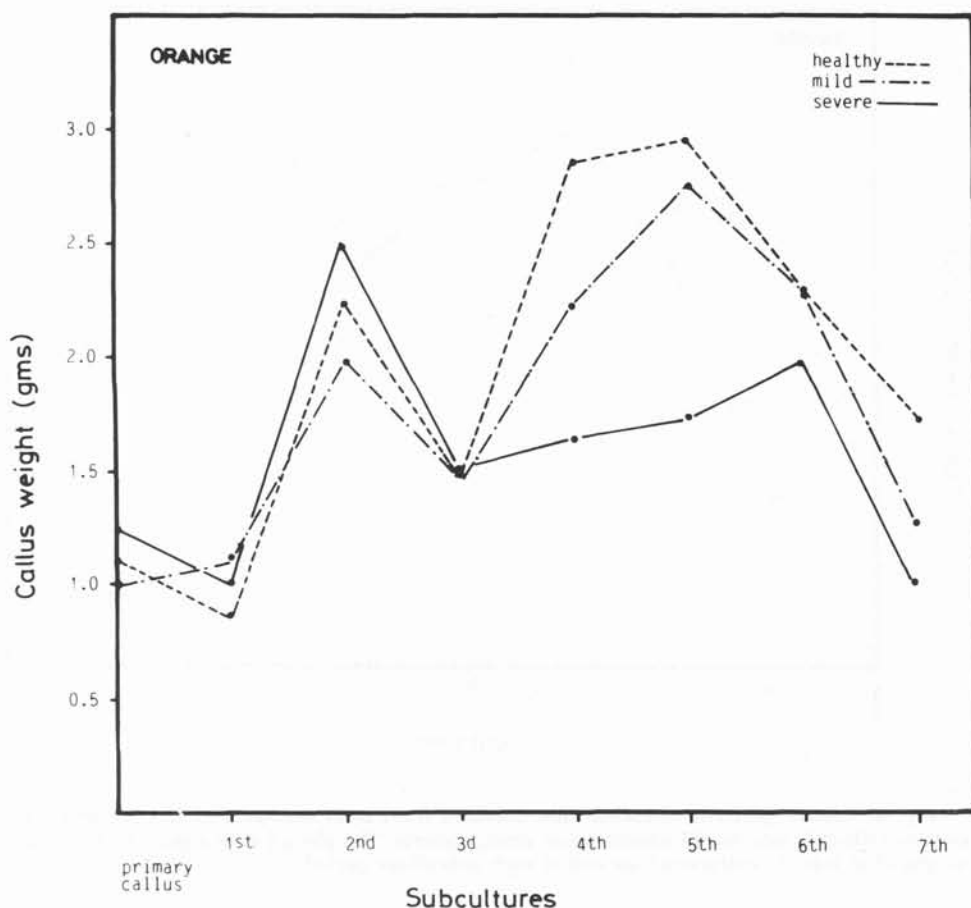


Fig. 6. Growth patterns of callus lines obtained from stem segments of healthy and psorosis-infected (severe P-121 and mild P-123 strains) Pineapple sweet orange plants. The data plotted represent the mean weight of at least 15 cultures at the end of each subculture period.

VIRUS CONTENT

The callus lines maintained by subculturing were periodically indexed to evaluate the presence of virus and viruslike pathogens. Callus derived from CTV- and CIVV-infected plants were tested by serology and callus derived from psorosis, vein enation and cachexia were indexed by graft inoculation to indicator plants.

The ELISA-double antibody sandwich (ELISA-DAS) test (4) was performed at the end of each 4-week subculture. The callus samples weighed 100-300 mg each. Tissue samples from healthy and infected mother plants growing in the greenhouse were used as negative and positive controls. The polyclonal anti-

serum 879 (kindly provided by S. M. Garnsey, USDA, Orlando, Florida) and the monoclonal antiserum 3DF1 (25) were used in CTV tests, and the polyclonal antiserum 997-FCVV-1 (also provided by S. M. Garnsey, USDA, Orlando, Florida) was used in CIVV tests. The immunoglobulins used were purified following Cambra *et al.* (3).

Biological indexing was done essentially as described for indexing citrus trees (1). Inoculation was performed by introducing a piece of callus underneath the bark (fig. 7), and incubation and reading of symptoms followed the system described for indexing of trees (1).

Tristeza. Callus lines originated from healthy and tristeza-infected

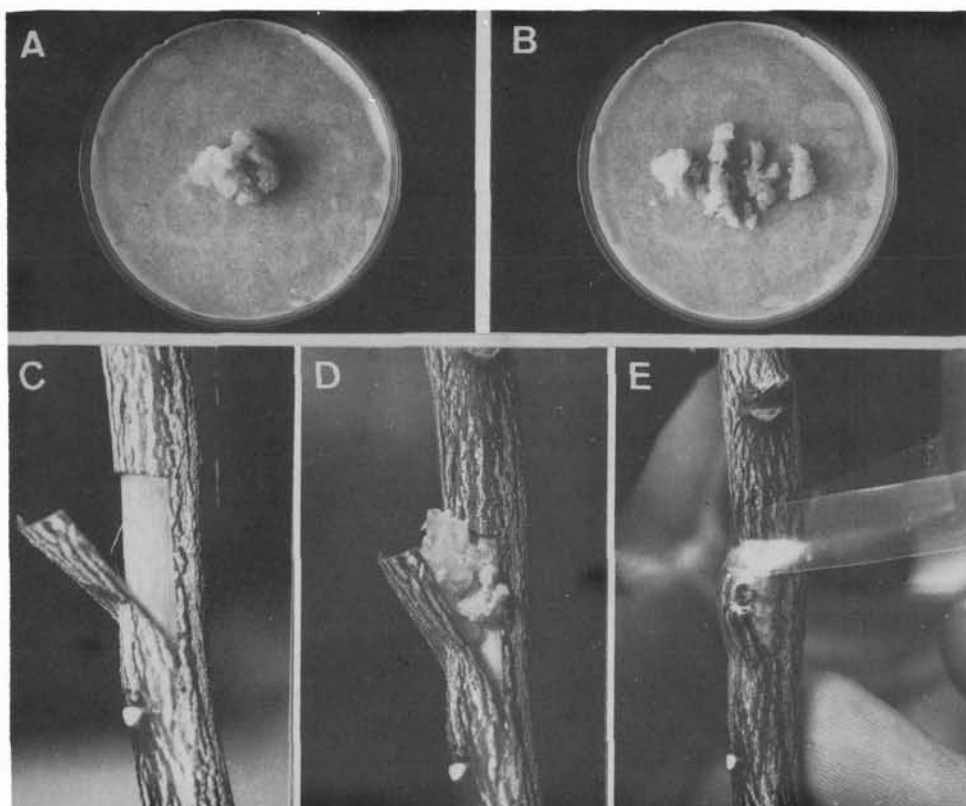


Fig. 7. Biological indexing of callus tissues. A) callus to be indexed B) callus divided into smaller portions. C) incision done on the indicator plant D) callus introduced under the bark E) wrapping of graft.

Pineapple sweet orange and Mexican lime tissues were tested at the end of each 4-week subculture. The number of calli indexing positive decreased in each subculture. Virus was not detected at the end of the third subculture in Pineapple sweet orange callus, and at the end of the fifth subculture in Mexican lime callus (table 2).

These results, as well as the low OD readings (measured at 405 nm) in ELISA plates indicated that CTV antigen was present in low amounts and that CTV did not replicate well in callus cultures. Whether the positive readings obtained during the initial subcultures were the result of actual virus replication or were simply due to the presence of virions or coat protein from the original explant, cannot be deduced from the data. In fact, the absence of vascular bundles on long-term established callus cultures

would prevent the spread of tristeza particles which are known to be mainly restricted to the phloem. The finding that severe strains of CTV were able to invade the ground meristem adjacent to protophloem of susceptible varieties (19), suggests that it may still be feasible to select cell and callus lines able to replicate CTV. However, further selection of callus lines capable of replicating tristeza was beyond the scope of this study.

Infectious variegation. Callus lines originating from healthy and CIVV-infected Pineapple sweet orange and Arizona 861-S-1 citron tissues were tested at the end of each subculture period. All Arizona 861-S-1 citron callus lines indexed positive and the OD readings were comparable to the tests of the mother plant tissue. In addition, the OD readings did not diminish in the six subcul-

TABLE 2
DETECTION BY ELISA-DAS OF TRISTEZA AND INFECTIOUS VARIATION IN
CALLUS CULTURES AFTER PERIODICAL SUBCULTURING

Source of tissue			Number of calli indexing positive (%) ^a					
			Subculture					
Disease	Host	Isolate no.	1st	2nd	3rd	4th	5th	6th
Tristeza (CTV)	Pineapple sweet orange	Control	0	0	0	0	0	—
		T300	50	21	0	0	0	—
		T308	50	36	0	0	0	—
	Mexican lime	Control	0	0	0	0	0	—
		T300	37	40	20	0	0	—
		T308	39	35	16	19	0	—
Infectious Variegation (CIVV)	Pineapple sweet orange	Control	0	0	0	0	0	0
		IV-400	100	100	100	90	60	60
	Arizona 861-S-1 citron	Control	0	0	0	0	0	0
		IV-400	100	100	100	100	100	100

^aIn each subculture at least 15 calli per treatment were indexed.

tures done over a 6-month period. These results indicate that CIVV replicates well on callus cultures. The number of callus indexing positive decreased in the sweet orange callus lines, although the OD readings remained high in the cultures indexing positive. Therefore, recovery of callus lines that indexed negatively was probably due to uneven distribution of the virus in the tissue rather than lack of replication. Callus cultures containing CIVV were kept in culture over 1 year.

Psorosis. Callus lines originating from healthy and psorosis-infected tissues were indexed biologically using Pineapple sweet orange and Dweet tangor seedlings as indicators. Grafting success and survival of the inoculum callus could not be evaluated by gross observation, however, a very high incidence of transmission was obtained (table 3) with calli from P-121 and P-123 sources. In all cases the symptoms observed in the indicator plants were the same as those in the mother plants.

Vein enation. Callus lines from vein-enation-infected tissues were indexed biologically utilizing Mexican lime seedlings as indicator plants. Only 3 of the 20 calli from the first

subculture indexed positive and none of the 19 calli indexed after the fifth subculture induced any reaction on the indicators (table 3). Whether the negative results obtained were the result of poor transmission of the disease agent from callus tissue or due to its absence, could not be determined.

Cachexia. Callus lines from citron infected with cachexia were indexed utilizing Parson's Special mandarin grafted on rough lemon rootstock (17). Eight months after inoculation, nine of the 10 calli indexed had induced severe gumming at the budunion of the indicator plants. These fast and conclusive results indicate that the cachexia disease agent is present, and probably at high titer, in callus cultures from the first subculture. Knowledge that the disease agent is a viroid (23) provides new tools for determination of its presence in callus cultures.

CONCLUSIONS

Tissue culture provides a system for the study of host-pathogen interaction under relatively controlled conditions. The data presented in this report indicate that the utilization of callus cultures derived from infected

TABLE 3
DETECTION BY BIOLOGICAL INDEXING OF PSOROSIS, VEIN ENATION AND
CACHEXIA IN CALLUS CULTURES MAINTAINED OVER PERIODICAL SUBCULTURING

Disease	Source of tissue		Number of calli indexing positive (%) ^z	
			Subculture	
	Host	Isolate no.	1st	5th
Psorosis	Pineapple sweet orange	Control	0	0
		P-121	89	70
		P-123	84	90
Vein Enation	Pineapple sweet orange	Control	0	0
		VE-207	15	0
Cachexia	Arizona 861-S-1 citron	Control	0	—
		X-704	90	—

^zIn each indexing test at least 10 calli per treatment were indexed.

tissues has potential in citrus virology.

The determination of the virus content of callus cultures initially infected with different disease-causing agents illustrates the different responses of callus to infection with citrus virus and viruslike pathogens. The CTV, a closterovirus mainly restricted to the phloem, and the vein enation agent cannot be detected in callus cultures after a few subcultures. This behavior is similar to the results reported with tissue cultures initiated with TMV-infected tissues (2, 12, 21, 22) and limits the use of callus cultures for the study of these diseases.

Conversely, callus lines established with CIVV-infected tissues seem to maintain stable virus titers over successive subculture. The CIVV-infected callus lines are being studied (definition of purification techniques, electron microscopy of infected cells, and response to modification of culture media and environment) to define a tissue culture system for research on this virus. Preliminary results indicate that, although healthy and CIVV-infected callus look alike in terms of gross morphology, infected cultures display characteristic malformations at the cell level when studied by electron

microscopy. Attempts to purify CIVV from callus revealed that purification of virus particles could be easily achieved with yields comparable to or higher than those from plant tissues.

Callus lines also perpetuate psorosis and cachexia pathogens and are being utilized as a tissue source for the study of the agents of these diseases.

In summary, callus cultures provide a tissue culture system for the study of CIVV, psorosis and cachexia-xyloporosis diseases. These calli display no symptoms in growth morphology and growth rates under the standard conditions of temperature and medium utilized. They are easy to grow, maintain, manipulate and store and, therefore, may become a useful tool for the study of these diseases.

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